

Kin discrimination and altruism in the larvae of a solitary insect

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Kin selection theory predicts altruism between related individuals, which requires the ability to recognize kin from non-kin. In insects, kin discrimination associated with altruistic behaviour is well-known in clonal and social species but in very few solitary insects. Here, we report that the solitary larvae of a non-social insect *Aleochara bilineata* Gyll. (Coleoptera; Staphylinidae) show kin discrimination and sibling-directed altruistic behaviour. Larvae superparasitize more frequently the hosts parasitized by non-kin individuals than those hosts parasitized by siblings. Kin discrimination probably occurs by self-referent phenotype matching, where an individual compares its own phenotype with that of a non-familiar related individual, a mechanism rarely demonstrated in animals. The label used to recognize kin from non-kin corresponds to substances contained in the plug placed on the hosts by the resident larvae during the parasitization process. Kin competition induced by a limited larval dispersion may have favoured the evolution of kin recognition in this solitary species.

Keywords: kin discrimination; self-referent phenotype matching; green beard effect; *Aleochara bilineata*; label; superparasitism

1. INTRODUCTION

Based on kin selection theory, a genetic trait may be selected even if it does not give an advantage to the individual (i.e. *direct* fitness), provided that it sufficiently increases the fitness of individuals sharing its genes (i.e. *inclusive* fitness). Altruism will be favoured when Hamilton's rule $rb - c > 0$ is satisfied, where b is the fitness benefit to the recipient, c the cost for the altruist and r their genetic relatedness (Hamilton 1963, 1964). Kin discrimination mechanisms frequently used by animals seem to require complex learning abilities, which are usually restricted to the adult stage because of the cost of evolving a recognition system (Gamboa *et al.* 1986, review in Waldman 1988).

In insects, kin discrimination which leads to the evolution of altruism seems to require either a strong genetic similarity between individuals (clones), or two evolutionary pioneer stages of insect societies: (i) group formation of cooperating individuals and (ii) interference from reproductive individuals leading to the formation of castes of non-reproductive individuals (workers and soldiers) (Alexander 1974; Andersson 1984; Gadagkar 1990; Thorne 1997; Giron *et al.* 2004). However, a simple increase of kin encounters through limited dispersion (i.e. viscous population) can theoretically lead to the evolution of behaviours which disadvantage the individual's *direct* fitness to the benefit of kin individuals recognized as such (i.e. *inclusive* fitness) (Hamilton 1964; van Baalen & Rand 1998). Nevertheless, the role of viscosity is controversial in the literature because it can also increase kin competition,

which might reduce or cancel the benefits of altruistic behaviours and therefore its selective advantage (West *et al.* 2001, 2002).

The larvae of *Aleochara bilineata* (Coleoptera; Staphylinidae) develop as pupal parasitoids of cyclorrhous flies including the cabbage root fly, *Delia radicum* (Colhoun 1953). In contrast to the great majority of hymenopteran parasitoids, *A. bilineata* females do not lay their eggs directly in or on a host but in locations likely to harbour hosts. First instar larvae must then actively search for a host (Fournet *et al.* 2001). After finding a suitable host, the larva gnaws its way through the hardened puparium and settles in the space between the puparium and the nymph. Then, it closes the entrance hole by excreting a viscous substance through the anus, which serves as a plug (Fuldner 1960). The time necessary for the piercing and plugging processes is highly variable (12–36 and 6–12 h, respectively) (Colhoun 1953, Fuldner 1960). To potentially recognize the relatedness of larvae already present in a host, foraging larvae can use cues either originating from the resident larva and perceptible through the host puparium, present in the plugging substance or deposited by the resident larva on the puparium during host handling.

First instar *A. bilineata* larvae are both time and energy limited since their longevity before feeding on the host is on average 4 days, and they cannot feed before parasitizing a host. They are small (1.62 ± 0.01 mm) and wingless; consequently, their movements are restricted to a limited volume of soil around their birthplace. Their *D. radicum* hosts are strongly aggregated in the field: the root system of one plant can contain 15–40 larvae and pupae while the next plant on the same crop row might not show a single

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host (Coaker & Finch 1971). Owing to host aggregation and larval biology, foraging for a host remains very local. Since *A. bilineata* females lay eggs in clutches, foraging larvae are likely to encounter hosts parasitized by sibs. In this system, viscosity and kin competition are linked since the less the larvae disperse the more likely they are to find hosts already parasitized by sibs. Yet, only one larva can develop per host; any host intruder being attacked by the resident larva until only one competitor remains or both die (Fuldner 1960). Foraging larvae are capable of discriminating between healthy and parasitized hosts, and avoid the latter when given enough host choice, time and physiological reserves (Royer *et al.* 1999). Accordingly, larvae capable of kin recognition should discriminate kin from non-kin and more frequently avoid hosts parasitized by a kin than hosts parasitized by an unrelated larva. When larvae hatch in a poor quality patch, where most hosts are already parasitized, their chances of finding a suitable host are very low and their mortality by starvation is likely to be high. If larvae more frequently avoid hosts parasitized by sibs than hosts parasitized by non-relatives, their mortality should increase as the patch contains more sib-parasitized hosts. We predict that larval mortality due to starvation will be higher when the larvae find only hosts parasitized by sibs and decrease when they find hosts parasitized by non-relatives or unparasitized hosts.

In this study, we addressed the kin discrimination ability of *A. bilineata* larvae by offering them the choice between a host parasitized by an unrelated conspecific larva and another host parasitized by a sib. To investigate fitness costs related to choices made by larvae in poor quality patches, we measured the percentage of larvae which did not parasitize any host depending on the parasitism status of the hosts encountered (healthy, parasitized by a conspecific, parasitized by a sib). In our experimental set-up, these larvae died from starvation since no other hosts were available. Finally, we investigated whether the kin recognition label was deposited by the first larva during host handling or related to the plugging substance used by the larva to close the entrance hole it gnaws through the puparium.

2. MATERIAL AND METHODS

(a) *Host and parasitoid rearing*

Delia radicum fly populations (host) were established in the laboratory from field-collected flies during the summer of 1994 at St Méloir des Ondes (Brittany, France). The brood was reared using the Neveu *et al.* (1996) method. The *A. bilineata* strain was also established using individuals collected during the summer of 2002 in St Méloir des Ondes. They were reared according to the Hertveldt *et al.* (1984) method. Both strains are refreshed yearly with new field-captured individuals coming from the same population.

The parents of the larvae used in the experiments were obtained as follow: parasitized pupae were isolated in an Eppendorf tube (Kartell Spa, Noviglio (Milan), Italy; height: 3.8 mm; diameter: 10 mm) until adult emergence. Emergences were checked every 24 h, and virgin adults were placed together in order to observe mating. After mating, each pair of parents was isolated in an aerated circular dish (Caubère, Yèbles, France; height: 25 mm; diameter: 80 mm) with food *ad libitum*. Two black pieces of cloth were water

dampened and placed at the bottom of the dish. Eggs were laid on the cloth and collected twice a week. Each egg was isolated in a Beem capsule (Agar Scientific, Essex, England; height: 1.4 mm; diameter: 0.7 mm) according to the family it came from. Hatched eggs were checked daily and larvae remained isolated until the experiment. Only first instar larvae aged less than 24 h were used in the experiments. A total of 30 different couples of parents was used in these experiments.

(b) *Parasitization procedure*

To obtain parasitized hosts, we placed a healthy *D. radicum* pupa in moist sand in a small dish (Caubère; height: 8 mm; diameter: 26 mm). A 0–24 h old larva (related or unrelated to the resident larva) was then placed on top of the sand, and the dish was closed. The process was stopped after 48 h by removing the pupa. The pupa was then rapidly washed in water using a paintbrush and checked under a dissecting microscope to assess the parasitization status. Only hosts parasitized by first instar larvae were used. The parasitization procedure was the same for all experiments, except for some variations in the last two experiments that are detailed later.

(c) *Experimental set-up*

For all experiments, two hosts (healthy and/or parasitized) were placed horizontally on a thin layer of moist sand in a small dish. The two hosts were then covered by moist sand (1 vol. water/4 vol. sand) up to the top of the dish. A 0–24 h test larva was deposited at the centre of the dish at an equal distance from the two buried hosts, and the dish was closed. The experiment was stopped after 96 h, the pupae cleaned and the hosts were observed as described earlier to count the larvae present in each host. Since the lifetime of *A. bilineata* first instars is on average 96 h, any unrecovered larvae were considered to have starved to death at that time.

Experiment 1: kin discrimination. In this experiment, two parasitized hosts were used, one parasitized by a sib (sisters and/or brothers born from the same parents) and the other by an unrelated larva (born from different parents).

Experiment 2: starving mortality. Mortality was studied in larvae given the choice between (A) a healthy host and a host parasitized by an unrelated larva, (B) a healthy host and a host parasitized by a sib, (C) two hosts parasitized by unrelated larvae and (D) a host parasitized by an unrelated larva and a host parasitized by a sib.

Experiment 3: host marking. In order to check whether an external host-marking behaviour could take place before the plugging process, we used hosts in which the entrance hole had not been plugged by the resident larvae. Parasitized hosts were put in water and washed for 3 min using a brush. Two treatments were compared: water-washed and unwashed puparium.

Experiment 4: plugging substance. Two situations were studied in order to estimate the role of the plugging substance in the recognition process: (i) hole closed, where resident larvae had at least started to plug their entrance hole and (ii) hole still completely open, where resident larvae did not initiate the plugging process. We prepared the two sets of hosts by observing pupae 48 h after initiation of parasitization and placing them in the experimental set-up either before or after the larvae had started to plug their entrance hole. The second and fourth experiments were carried out at the same time, using the same parents.

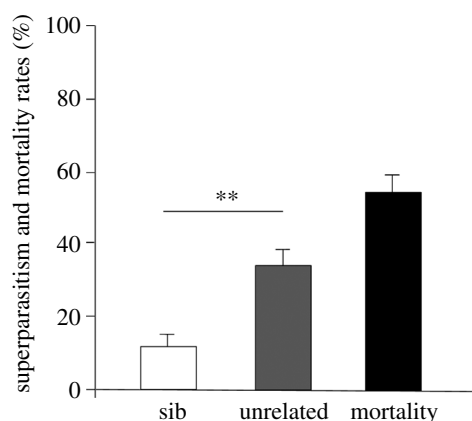


Figure 1. Kin discrimination. Superparasitism rate of hosts parasitized by siblings (white bar) or unrelated larvae (grey bar) and mortality rate (black bar) ($n=100$). NS: not significant, $**p<0.01$ (G -test), error bars: standard errors.

(d) Statistical analyses

Analyses were performed using R 2.2.1 (Ihaka & Gentleman 1996). Superparasitism levels and mortality levels were compared by a G -test. Mortality levels in experiment 2 were analysed globally with a G -test and between treatments with a generalized linear model (GLM) procedure assuming a binomial error distribution with a logit link function (McCullagh & Nelder 1989). Differences in responses under different treatment levels were assessed by analysis of deviance using a χ^2 -test (ANOVA (GLM) procedure).

3. RESULTS

(a) Kin discrimination

The larvae more frequently avoided hosts containing a sib than hosts containing an unrelated competitor (figure 1). Out of the 46 larvae that entered into a contest with a resident larva, 34 (74%) competed with an unrelated resident larva rather than a sib ($G=10.96$, 1 d.f., $p<0.001$). In this experiment, numerous larvae died of starvation since 54 out of the 100 larvae tested did not enter any host.

(b) Starving mortality

A global comparison showed significant differences between mortality rates ($G=42.00$, 7 d.f., $p<0.0001$) (figure 2). When test larvae had a choice between a healthy and a parasitized host, the mortality rates remained low and did not differ significantly, whether the parasitized host harboured a kin or an unrelated larva (GLM deviance analysis, A versus B, $p=1.00$). Mortality increased significantly when both hosts were parasitized (A versus C, $p=0.02$; A versus D, $p<0.0001$). However, mortality was significantly higher when one of the two hosts was parasitized by a sib compared to the situation where both hosts were parasitized by a non-relative (C versus D, $p=0.02$).

(c) Host marking

Superparasitism rates were similar, whether parasitized hosts had been water-washed or not ($G=0.17$, 3 d.f., $p=0.98$) (figure 3). The larvae therefore did not recognize kinship using a water soluble external mark potentially

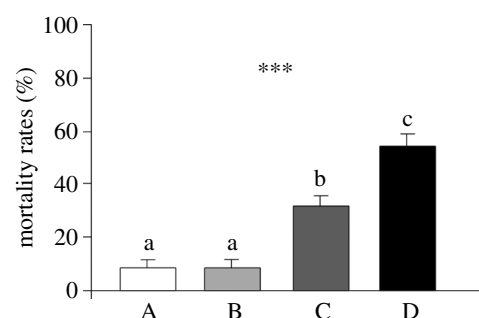


Figure 2. Starving mortality. Mortality rates observed when larvae were given the choice between: (A) an unparasitized host and a host parasitized by a non-relative ($n=35$), (B) an unparasitized host and a host parasitized by a sib ($n=35$), (C) two hosts parasitized by non-relative ($n=35$) and (D) a host parasitized by a non-relative and a host parasitized by a sib ($n=100$). $***p<0.001$ (G -test), error bars: standard errors. Within treatments, bars with different letters are significantly different ($p<0.05$) from each other (ANOVA (GLM) and χ^2 -test).

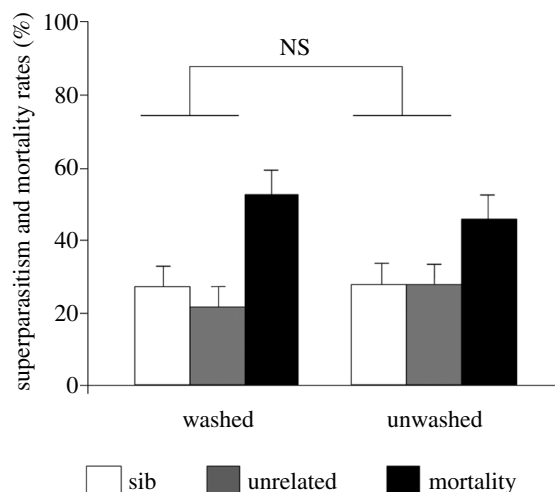


Figure 3. Host marking. Superparasitism rate of hosts parasitized by siblings (white bars) or unrelated larvae (grey bars) and mortality rate (black bars) depending on whether the parasitized hosts were water-washed before the experiment ($n=55$), or unwashed ($n=55$). NS: not significant (G -test), error bars: standard errors.

deposited by the resident larva on the puparium during host handling.

(d) Plugging substance

Superparasitism rates differed according to the presence or absence of the plugging substance, excreted by the resident larva during entrance hole closure ($G=17.07$, 3 d.f., $p<0.001$) (figure 4). When the resident larva did not have the time to excrete the plugging substance, superparasitism was not differently oriented towards hosts occupied by sibs or unrelated resident larvae ($G=0.96$, 1 d.f., $p=0.32$), indicating that kinship was not detected by foraging larvae. In contrast, when resident larvae had excreted the plugging substance, foraging larvae strongly avoided penetrating hosts occupied by a sib while accepting preferentially a host harbouring an unrelated resident ($G=20.57$, 1 d.f., $p<0.0001$). The percentage of larvae dying from starvation did not differ significantly, whether the hole was plugged or not ($G=2.41$, 1 d.f., $p=0.12$).

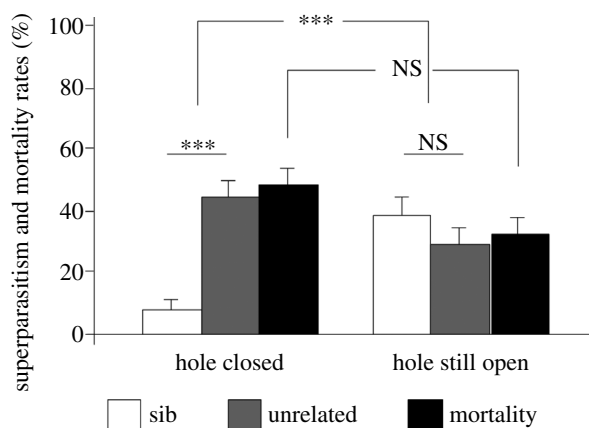


Figure 4. Plugging substance. Superparasitism rate of hosts parasitized by siblings (white bars) or unrelated larvae (grey bars) and mortality rate (black bars) depending on whether the entrance hole gnawed through the puparium by the resident larvae had been closed (hole closed, $n=75$) or not (hole still open, $n=75$). NS: not significant, $**p<0.01$ and $***p<0.001$ (G-test), error bars: standard errors.

4. DISCUSSION

Our results demonstrate that first instar *A. bilineata* larvae are capable of kin discrimination, since they mainly superparasitize hosts containing unrelated larvae, avoiding hosts parasitized by sibs. Furthermore, we have located the label used by these larvae to recognize their sibs inside parasitized hosts. Larvae use neither the external marks potentially deposited by the first larvae on the puparium during host handling, nor the volatile compounds diffusing through the puparium. Instead, they use a label (probably chemical), correlated with the plugging substance, excreted by the resident larva during entrance hole closure of the host puparium. Finally, the kin recognition mechanism used by *A. bilineata* larvae may rely on a self-referent phenotype matching mechanism or on recognition alleles (i.e. the green beard effect).

(a) *Aleochara bilineata* larvae exhibit kin recognition and altruism towards their sibs

Our experiments demonstrate that first instar *A. bilineata* larvae recognize their kin, since they superparasitized preferentially the hosts harbouring an unrelated larva rather than those parasitized by a sibling. Considering that superparasitism in this solitary species leads to a fight to the death with the resident larva, the stronger avoidance of relatives may be interpreted as altruistic behaviour towards kin because (i) it is obviously beneficial to the resident sib and (ii) it represents a cost to the foraging larva, which has to keep foraging for another host. In our experimental conditions, a greater mortality of the larvae avoid super parasitizing hosts already parasitized by sibs was observed, confirming altruism. Such behaviour is remarkable when considering that the larvae were placed in a small structure with two easily accessible hosts. Larval mortality varied according to the parasitism status of the hosts proposed. This mortality was higher when sib-parasitized hosts were present and lower when healthy hosts were present.

Within the framework of Hamilton's kin selection theory, altruistic behaviour can only be selected if $rb - c > 0$ (r is the coefficient of relatedness, b the benefit to the recipient and

c the cost to the actor). Since here $r=0.5$ (full sibs) the observation of altruistic behaviour suggests that $b > 2c$, meaning that either the benefit to the resident is high (the resident would often be killed by a non-altruist larva) or the cost to the altruist is low (it is easy to find another host). Marking experiments would be necessary to determine the mortality rates of both larvae in case of superparasitism and thus to calculate b . Calculating c would require a fine knowledge of the host finding chances of the larvae in natural conditions. However, since hosts are strongly aggregated in the field, finding a host could be a strong predictor that other hosts are present nearby and thus c might not be very high.

(b) The kin recognition label is associated with the plug obstructing the entrance hole

Kin recognition did not occur in our experiments when the resident larva had not plugged its entrance hole. In contrast, kin recognition was very clear when the plug was in place, even partially. These results demonstrate that the kin recognition mechanism used by *A. bilineata* relies on substances associated with the plug obstructing the entrance hole of the resident larva. It does not depend on a cue that would be perceived through the puparium or deposited on the puparium before host penetration, since there was no kin recognition when the entrance hole was still completely open. Moreover, the experiment with water-washed hosts shows that the prospecting larvae do not recognize their kin by using external water-soluble marks potentially deposited on the puparium by the resident larva during the host handling process.

Kin recognition also took place in our very first experiment in which we had not checked whether the plug was present or not (hole open or already closed). However, in this earlier experiment, the resident larvae had been given ample time (48 h) to parasitize a healthy host. In a recent experiment, we found that 70% (95% CI: 66–74) of resident larvae had closed their entrance hole within 48 h. We can thus assume that most entrance holes were plugged when the foraging larvae were released in the experimental set-up, thus explaining why kin recognition was made possible.

(c) Phenotype matching mechanism or 'green beard effect'?

Following the definitions of Waldman (1988) and Holmes (2004), kin recognition can be either *direct*, when recognition is based on kin encounters, or *indirect*, when kin recognition is based on contexts, such as spatial location (e.g. Buczkowski & Silverman 2005). *Direct* kin recognition can be mediated by three mechanisms: (i) social learning (or prior association), (ii) phenotype matching and (iii) recognition alleles allowing the 'green beard effect' (Dawkins 1976; Blaustein 1983). Social learning refers to interactions between individuals resulting in familiarity regardless of genetic relatedness: any individual will become familiar if it is encountered often, whether it is a relative or not. Only the last two mechanisms whereby an individual recognizes (recognition alleles) or learns the phenotypic cues of its siblings or its own cues (phenotype matching) permit true genetic recognition since phenotypic cues reflect the individual genotype (Gadagkar 1985). To estimate the genetic relationship (r), kin recognition requires genetically determined cues. Therefore, recognition templates should

be based on individual characters (Gadagkar 1985; Sherman *et al.* 1997). In the particular case where the individual learns its own cues to recognize its kin (without any prior encounter with kin individuals), the mechanism is called 'self-referent phenotype matching' (Hauber & Sherman 2001; Holmes 2004).

Social learning and phenotype matching seems to have been adopted by the great majority of animals recognizing kin (Waldman 1981, 1982; Porter *et al.* 1983; Gadagkar 1985; Getz & Smith 1986; Sun & Müller-Schwarze 1997; Mateo & Johnston 2000). Self-referent phenotype matching and recognition alleles concern a few examples of kin recognition, probably not due to their rarity but because they are so difficult to demonstrate (Mateo & Johnston 2000; Jansen & van Baalen 2006).

The larvae interacting in our experiments had not previously encountered conspecifics: they had hatched and were maintained in complete isolation until the experiments. Neither social nor environmental influences from parents or sibs can thus explain the kin discrimination mechanism used and also the following decisions. Therefore, *A. bilineata* larvae could either use a self-referent phenotype matching mechanism, or recognition alleles. Further experiments removing larval odours or blocking their possible learning ability are needed to distinguish between the two kin recognition mechanisms that could be used.

(d) *Aleochara bilineata* larvae recognize their kin using an endogenous label

The cues used to recognize kin from non-kin may be visual, auditory or olfactory. Kin recognition in social insects commonly relies on olfactory cues associated with epicuticular lipids (Breed & Bennett 1987). Olfactory cues can be acquired exogenously from food and communal nesting materials, but they can also be produced endogenously either through cytoplasmic transfer of maternal cues or produced by the individual itself (Downs & Ratnieks 1999). However, even genetically determined cues can change according to social and ecological conditions. For instance, colony odours of many social insects (Breed & Bennett 1987) and recognition odours of some mammals (Leon 1983) vary in response to changes in food or nesting materials. In our experiment, considering that all mothers received identical food *ad libitum* and that *A. bilineata* larvae do not eat before feeding on the host, the kin recognition label cannot be linked to the kind of food consumed by the mother, to a common food consumed by sibs or to microhabitat. The label therefore might be endogenous since it does not depend on food or rearing in common nest conditions as usually found in social insects (Waldman 1988; Holmes 2004). Accordingly, we can conclude that sib discrimination in *A. bilineata* larvae is a *direct* kin recognition mechanism, each larva being able to recognize odours closely matching its own, through a self-referent phenotype matching mechanism or recognition alleles. Whether the label is transmitted by the mother or self-produced by the larvae can be assessed in recognition experiments between cousin larvae coming from fathers that are brothers. If such larvae recognize their cousins and avoid superparasitizing them, this would demonstrate that the kin recognition label is self-produced and not provided by the mother, since these cousins have different mothers.

(e) Possible origin of kin recognition in a solitary parasitoid species

The benefits of kin recognition in a particular species depend on its life history and the kind of decisions it involves. Gadagkar (1990) argued that in eusocial insects, kin recognition is not required before two evolutionary stages are reached: (i) group formation of mutualistic individuals (i.e. 'the gambling stage') and (ii) reproduction usurpation or parental manipulation and subfertility (i.e. 'the manipulation stage'). In *A. bilineata* larvae, which are not social, neither group formation of mutualistic individuals nor reproduction manipulation can explain the evolution of a kin recognition mechanism. However, the frequency of encounters between larvae and kin-parasitized hosts (due to population viscosity) seems sufficient to have allowed kin discrimination to evolve, leading to altruistic behaviour between siblings. Yet, population viscosity also tends to increase kin competition. According to West *et al.* (2001, 2002), an excessively high level of kin competition may even cancel-out the benefits of an altruistic behaviour. In the lizard *Lacerta vivipara*, population viscosity and kin competition are in fact reduced by kin discrimination, which increases dispersion. In this species, juveniles are more repulsed by the odour of their mother than by that of a foreign female (Léna *et al.* 2000). Such a behaviour could also occur in *A. bilineata* and it would be interesting to study the influence of a higher level of kin competition on *A. bilineata* dispersion. However, our results suggest that the level of competition is high enough in natural conditions to have selected for a kin discrimination mechanism, but low enough so that it still pays to be an altruist towards siblings.

In conclusion, the present results describe for the first time a kin discrimination ability in solitary parasitoid larvae. These results are particularly exceptional in a non-social species where neither family groups (offspring, or adults and their offspring) nor groups of unrelated individuals have ever been described. The evolutionary pressures explaining kin selection evolution leading to altruistic behaviour (kin competition deriving from viscosity) are different from those invoked in social and clonal insects.

In the near future, we hope to chemically identify the kin recognition label present in the plug, and to define whether this label and the mechanism are potentially genetically transmitted from one or both parents. Investigation of the extent to which kin discrimination can be achieved regarding the levels of competition and relatedness may also be useful to determine the origin of the establishment of kin discrimination in *A. bilineata*. Kin competition forces may have selected for kin discrimination, or kin discrimination may allow larvae to achieve adequate dispersion.

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